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Protein Structure and Function: Design and Synthesis of Peptide Recognition Sequences

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The goal of this research is to characterize the chemical and physical properties that define the interactions between the Arg-Gly-Asp (RGD) recognition sequence of protein adhesion molecules and their cellular receptors. NMR analysis of the solution conformation of the pentapeptide ligand GRGDS indicate a folded structure of the molecule in solution with a type II β-turn in which the amide carboxyl oxygen of Arg-2 receives a hydrogen bond from the amide NH of Ser-5. These studies are now being extended with a 14 residue ligand to test the role of the carboxylate and amino termini of the short peptide. The conformations of RGD peptides has also been analyzed by molecular mechanics and Monte Carlo methods. The predicted structure of the GRGDS molecule correspond to most of the NOE data. Studies of RGD binding proteins has led to the identification of a cell adhesion receptor corresponding to the Pgp-1/CD44/Hermes/SCMRIII family of molecules that are implicated in a variety of cell-cell adhesion processes. The cDNA of this protein has been isolated and sequenced and the molecule is being utilized as a possible in vitro model of RGD ligand-receptor binding.

keywords: molecular biology; receptor sites; binding sites; synthe...

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PROGRESS ON CONTRACT N00014-87-K-0344

PRINCIPAL INVESTIGATOR: Thomas August

CO-INVESTIGATORS: Mario Amzel
                          Albert Mildvan

CONTRACT TITLE: Protein Structure and Function: Design and Synthesis of
Peptide Recognition Sequences

START DATE: May 1, 1987

RESEARCH OBJECTIVE:

        Characterization of the chemical and physical properties that define
the interactions between the Arg-Gly-Asp (RGD) recognition sequence of
protein adhesion molecules and their cellular receptors.

PROGRESS (Year 2):

        The objectives during the second year of the program have been the
following:

        1. NMR Analysis of Peptide Structure and Conformation: The solution
conformation of the pentapeptide ligand GRGDS has been studies by one-and
two-dimensional proton NMR at 250 MHz. All of the proton resonances in the
spectrum of GRGDS have been assigned. Twelve interproton nuclear
Overhauser effects (NOE's) have been detected indicating interproton
proximities ranging from 2 to 5 A. Six of these twelve NOE's are
interresidual which provide detailed structural information, revealing
proximity between protons of Arg-2 and Ser-5, and between Arg-2 and Asp-4
(Fig. 1). The data indicate a turn or folded structure for GRGDS in
solution, and can be fit in detail by a type II β-turn in which the amide
carboxyl oxygen of Arg-2 receives a hydrogen bond from the amide NH of Ser-
5 (Fig. 2). Theoretical energy calculations reveal this structure to be
one of four low energy, hence stable conformations (1).

        To test the possibility that this bend might have resulted from the
presence of carboxylate and amino termini in this short peptide, we have

begun NMR studies of the solution conformation of the much longer, 14-residue RGD ligand, YAVT<u>GRGDS</u>PASSC, using our newly installed 600 MHZ NMR spectrometer. All of the proton resonances have been assigned by amino acid type based on expected chemical shifts and coupling patterns using phase sensitive double quantum filtered COSY and NOESY experiments. The assignments are, of course, sequence specific for the nonredundant amino acids. Additionally, the two Ala's were distinguished based on the expectation that Ala-2 NH would be broader due to chemical exchange with H_2O and more downfield at pH = 5 than Ala-11 NH. There are many NOE crosspeaks observed in the NOESY spectrum. The more structurally significant long range NOE's are summarized in Fig. 3. These preliminary results suggest a folded structure for the 14-mer as in the pentamer. However the type II β-turn appears to have shifted by two residues toward the carboxyl terminus, i.e. over the residues D_8-S_9-P_{10}-A_{11}. The chemical shifts of P_{10} are characteristic of trans proline.

The driving force for such a conformation could be an electrostatic interaction between the positive side chain of Arg-6 and the carboxyl terminus of Cys-14. Clearly the small and large RGD peptides are flexible and the location of the bends are both sequence dependent and length dependent. These observations make it all the more important to determine the conformation of the receptor bound ligand.

2. <u>Crystal structure and theoretical analysis of peptide conformation</u>: The conformations of tri-, tetra- and penta-peptides containing the RGD sequence were analyzed using Molecular Mechanics and Monte Carlo methods. A procedure was developed in which Monte Carlo methods are used for a) identifying the low energy conformations on the peptides and b) evaluating their thermodynamic properties. The peptides studied were RGD, GRGD, RGDG, GRGDG and GRGDS. For these peptides the initial search procedure found a series of stable structures that were then refined to minimize their energies. The structures found converge to those presented in Table 1. The value of the refined energy for each of the minima is indicated in the first column. In the second part all these minima were analyzed using a restricted Monte Carlo search that provided the average value of the energy and the heat capacity for each of the stable conformations. The structures obtained were analyzed with reference to the NMR data obtained in the laboratory of Dr. Mildvan. It was found that the structure T' of the pentapeptide GRGDS explained most of the NOE data and was one of the stable conformers determined in the Monte Carlo analysis. The combination of the theoretical calculations and the NOE data has been prepared for publication.

3. <u>Characterization of a cell surface receptor for RGD ligands</u>: A continuing effort is to develop an <u>in vitro</u> system for RGD ligand-receptor binding. We had previously purified platelet glycoprotein IIbIIIa, a known integrin receptor, in large quantities, but were unable to detect RGD peptide binding to the purified protein. This problem is attributed to their heterodimeric denaturation of the alpha and ß subunit structure of the receptor and the contribution of both subunits to the ligand binding domain. As an alternative approach we initiated experiments to determine if there was RGD binding activity of any receptor present in soluble extracts of cell membranes.

Detergent extracts of peripheral blood lymphocytes were passed over a RGD column and a putative RGD binding protein was demonstrated and identified as the CD44 cell adhesion glycoprotein. This molecule has recently been found to be homologous to the murine Pgp-1 differentiation alloantigen, the Hermes gp90 glycoprotein implicated in the binding of lymphocytes to high endothelial venules, and SCMRIII, a fibroblast glycoprotein that binds immobilized type 1 and type VI collagens. The broad tissue distribution of CD44/Pgp-1/Hermes/ECMRIII suggests that the protein plays a general role in cellular adhesion functions.

Our primary goal has been to determine the structure of this protein by cDNA sequence analysis and to initiate recombinant expression of the glycoprotein for high yield expression of the molecule in order to obtain sufficient quantity for <u>in vitro</u> binding studies, NMR analysis and X-ray crystallography. We have just completed the isolation and sequence analysis of a cDNA clone encoding the entire molecule. Interestingly, the protein shows sequence homology to cartilage link and proteoglycan core proteins and biochemical studies show that a small fraction of the protein contains covalently linked chondroitin sulfate glycosaminoglycan structures.

We also have discovered that monoclonal antibodies against the molecule cause cells to rapidly aggregate, providing direct evidence for the function of this molecule in cell adhesion. The natural ligand for the molecule and the processes involved in cell adhesion remain to be elucidated.

WORK PLAN (Year 3)

Synthesis and biological assay of RGD peptides (August): A series of ligands of fixed conformations due to disulfide bond formation are being investigated. Cyclic peptides of the following series are being synthesized, purified and analyzed for receptor binding _in vitro_ under reduced (linear) and oxidized (cyclic) conditions:

      CGRGDC
      CGRGEC
      CSGRCDSC
      CSGRGESC
      CSGRGDC
      CSGRGEC

Solution structure of ligands peptides (Mildvan and Vaughn): In the next year we plan to complete the solution structure of the 14-residue peptide ligand YAVTGRGDSPASSC, and refine it to the highest resolution possible. As a control, we will study the solution structure of the non-liganding peptide YAVTGRGESSPASSC to determine whether its failure to interact with receptors might result solely from the single substitution of E for D at position 8 or from more extensive structural changes.

We will also determine the solution structures of RGD peptides (and then RGE controls) in molecules containing cysteine residues at their amino and carboxy termini, in both their closed (oxidized) and open (reduced) conformations. The peptides to be studied are CSGRGDSC and CSGRGESC. Parallel receptor binding studies will enable us to correlate solution structures with biological activities.

RGD binding to CD44 glycoprotein (August): A major goal is to develop a sensitive and quantitative assay system to measure RGD binding to a soluble receptor; such as assay has not been described. Our current most efficient assay for RGD-receptor interaction is based upon RGD inhibition of cell binding to fibronectin. The affinity of RGD peptide for cell receptor in this assay is very low, requiring mM concentrations of RGD, and it is not practical to measure purified receptor-RGD binding in this system. Continued efforts are being made to obtain direct binding of receptor to RGD using solid phase assay systems and either radiolabeled receptor or a direct or indirect anti-receptor antibody system.

Crystal structure and theoretical analysis of peptide conformation
(Amzel and Cachau): The results of Year 2 suggest that hexa- and octa-
peptides containing cysteines at both the amino and carboxy termini, if
oxidized, will stabilize the T' conformation that was found by the
calculations and the NOE data. The peptides were synthesized and tested
for activity. We have been setting up all the programs necessary to do
calculations with cyclic peptides. We are going to start doing the
calculations on the hexa-and octa-peptides containing cysteines at both
termini and at the same time will attempt to crystallize the purified
peptides. It is well known that one of the problems in crystallizing small
peptides is their extreme flexibility. We expect that it will be much
easier to crystallize the cyclic peptides because cyclization greatly
restricts the number of accessible conformations.

INVENTIONS (Year 2)

None

PUBLICATIONS AND REPORTS (Year 2)

Publications in preparation

Cachau, R.E., Serpersu, E.H., Mildvan, A.S., August, J.T., and Amzel. M.L.
Recognition in cell adhesion: A comparative study of RGD containing
peptide conformations by Monte Carlo and 2D-NMR methods.

Belitsos, P.C., Hildreth, J.E.K., August, J.T. Leukocyte aggregation
induced by anti-CD44 (Pgp-1) monoclonal antibodies.

Wolffe, E.J., Holland, S.M., Gause, W., Steinberg, A., August, J.T.
Cloning and sequencing of mouse Pgp-1 (Ly24).

Abstracts

Lattman, E., and Amzel, M.L. Computer generated random confirmers of
polyalanine as a model for the unfolded state. Biophys. J., 55:20A (1989).

Cachau, R.E., and Amzel, M.L. Conformations of RGD containing peptides.
(Conference on Protein Structure and Drug Design, Erice, Italy, June,
1989).

Cachau, R.E., Serpersu, E.H., Mildvan, A.S., August, J.T., and Amzel, M.L. Recognition in cell adhesion: A comparative study of RGD containing peptide conformations by Monte Carlo and 2D-NMR methods. (Mid-Atlantic Protein Crystallographic Conference, Baltimore, May, 1989).

TRAINING ACTIVITIES

The following trainees have participated in this research program with funding provided from other sources.

1. Engin H. Serpersu, Ph.D. Postdoctoral Fellow, Biochemistry.
2. Joseph Vaughn, Ph.D. Research Associate, Biochemistry.
3. Peter C. Belitsos, M.D. Fellow, Pharmacology and Molecular Sciences.
4. Elizabeth J. Wolffe, predoctoral trainee, Pharmacology and Molecular Sciences.
5. Jeff Boyington, predoctoral trainee, Biochemistry, Cellular and Molecular Biology Training Program.
6. Ron Wang, predoctoral trainee, Biochemistry, Cellular and Molecular Biology Training Program.
7. Christopher K. Garcia, Predoctoral Fellow, Institute for Biophysical Studies on Macromolecular Assemblies.

Women or minorities - 1

Non-citizens:
    Engin Serpersu, Turkey, applied for permanent residence.
    Raul Cachau, Argentina
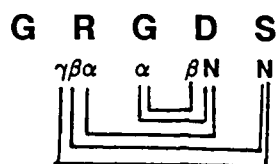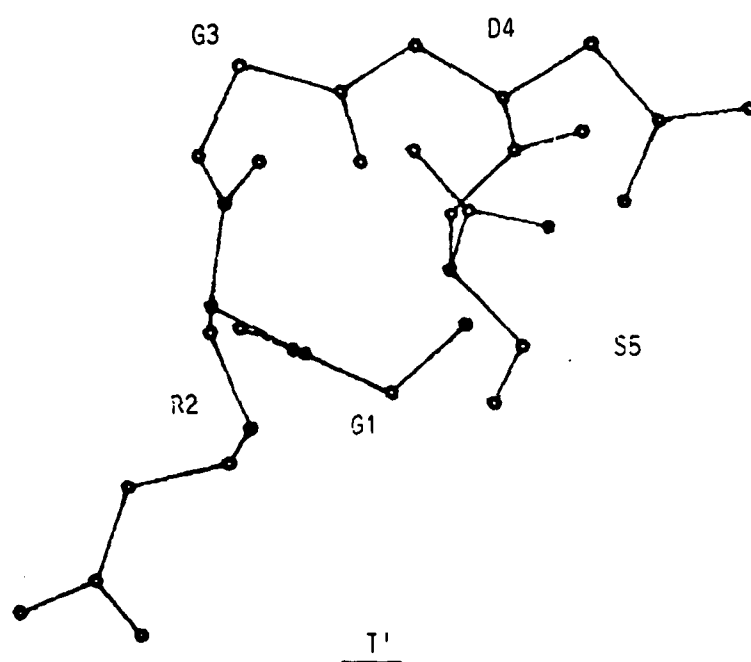   Ron Wang, Peoples Republic of China

G R G D S
γβα α βN N

Fig. 1 Long range NOE's in RGD pentamer.

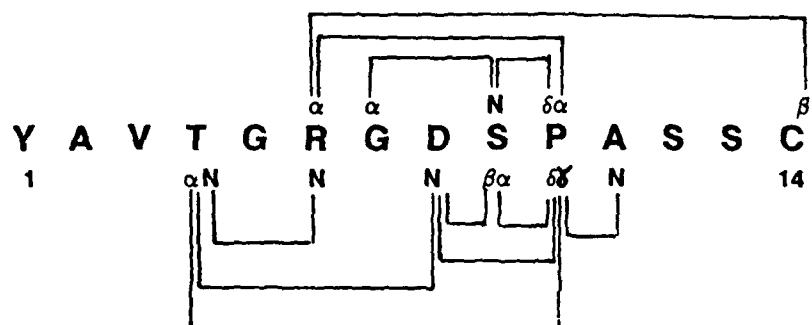Fig. 2 Low energy conformation of GRGDS consistent with NOE data.

Fig. 3 Long range NOE's in RGD 14-mer.

TABLE 1. CALCULATED THERMODYNAMICAL PROPERTIES OF RGD PEPTIDES

	Emin.	<E>	Cv	Struc. Description
Tripeptides				
a	-20.8	-15.5	0.50	open
d	-28.8	-27.2	4.34	closed
g	-25.9	-24.9	18.80	closed
Tetrapeptides				
1	-18.0			open
2	-25.1			closed
3	-24.1			closed
4	-27.7			closed
Pentapeptides				
A	-29.0	-28.2		closed
G	-27.0	-24.8		closed
T	-22.0	-20.9		closed
T'	-22.5	-21.2		closed